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Uses and Limitations of the XTT Assay in Studies of *Candida* Growth and Metabolism

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Colorimetric tetrazolium assays are used increasingly in studies of fungi, often in the absence of standardization or correlation with other methods. We examined species- and strain-related tetrazolium metabolism in *Candida albicans* and *Candida parapsilosis* by using XTT {2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide} and WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphonyl)-2H-tetrazolium] and found marked variations. Also, significant signal was often missed in the absence of dimethyl sulfoxide extraction.

Colorimetric assays of cellular viability are important tools in the study of eukaryotic cell activity. A mainstay of such techniques are assays involving the use of tetrazolium salts (1), which have evolved since the description of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in the early 1980s (13). The synthesis of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) (14) has proved especially useful, since the water solubility of its formazan product enabled the simplification of assay performance (15). Due to their ease of use, the MTT and XTT methods have been employed as assays of yeast viability (10, 11). Hawser et al. found that XTT could be used for fungal susceptibility testing (7). Recently, our group and others have used XTT to study fungal biofilm development and drug resistance (2, 4, 5, 8).

XTT is converted to a colored formazan in the presence of metabolic activity (the primary mechanisms of XTT-to-formazan conversion are the mitochondrial succinoxidase and cytochrome P450 systems, as well as flavoprotein oxidases [1]). Since the formazan product is water soluble, it is easily measured in cellular supernatants. This point is important in biofilm research because it allows the study of intact biofilms, as well as examination of biofilm drug susceptibility without disruption of biofilm structure (9). It is anticipated that XTT methods will be used increasingly to study fungal growth and drug susceptibility. Consequently, a better understanding of their uses and limitations is important.

Previous work using XTT to measure fungal activity showed a direct relationship between colorimetric signal and cell number (4). However, our group's previously published results (8, 9) and unpublished observations suggest that while this method is useful for comparisons involving one strain, its use may be more problematic in attempts to compare different fungal strains and species. This is an important caveat, since recent studies have made direct quantitative comparisons between fungal strains and species (4, 6), while data supporting the underlying assumption that all strains metabolize XTT in an equal fashion have not yet been published. Moreover, prior work examining the fungal metabolism of MTT provided limited data regarding the linearity of the *Candida* inoculum-formazan product curve; in fact, at high concentrations of some fungi (e.g., *Aspergillus fumigatus*), the curves became distinctly nonlinear (10).

To address these inconsistencies and to define the limits of the XTT assay, we undertook a comparative assessment of XTT metabolism among different clinical isolates, including *Candida albicans* and *Candida parapsilosis*. To ensure that our findings were not limited to one technique, we also examined the performance of a commercially available assay utilizing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphonyl)-2H-tetrazolium (WST-8) (12).

The *C. albicans* (M61 and GDH) and *C. parapsilosis* (P/A71, P92, and P177) strains used have been described previously (8). Organisms were grown in yeast nitrogen base (Difco Laboratories, Detroit, Mich.) supplemented with 50 mM glucose (2). Fifty milliliters of medium was inoculated with *Candida* cells from fresh potato dextrose agar (Difco) plates and incubated for 24 h at 37°C in an orbital shaker at 60 rpm. Cells were harvested, washed twice with 0.15 M phosphate-buffered saline (PBS; pH 7.2, Ca^{2+} and Mg^{2+} free), and resuspended in 10 ml of PBS. Blastoconidia were counted on a hemocytometer after serial dilution, standardized, and used immediately.

The XTT assay was performed as described previously (3, 7). Three-milliliter aliquots of standardized *Candida* suspension were transferred to the wells of 12-well tissue culture plates (Becton Dickinson, Franklin Lakes, N.J.). Fifty microliters of XTT solution (Sigma Chemical Co., St. Louis, Mo.) and 4 μl of menadione solution (1 mM concentration in acetone; Sigma) were added to each well. To determine how XTT concentration affected colorimetric signal, we used solutions at both 1 mg/ml (1×) and 5 mg/ml (5×) (both in PBS). Plates were incubated at 37°C for 5 h on a rocker table, and the suspension was agitated and pipetted from each well and centrifuged (5 min at 6,000 × *g*) to pellet cells. Formazan product in the supernatant was measured in terms of optical density at 492

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nm (OD_{492}) by using a spectrophotometer (Spectronic Genesys 5; Spectronic Instruments, Rochester, N.Y.). To determine whether a substantial proportion of formazan product was retained in cell pellets, the pellets were resuspended in 3 ml of 100% dimethyl sulfoxide (DMSO) and centrifuged and the OD of the supernatant was determined.

Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies Inc., Gaithersburg, Md.) uses WST-8 (12), which, upon bioreduction in the presence of the electron carrier 1-methoxy PMS, produces a water-soluble colored formazan. Twelve-well plates containing 3 ml of cell suspension/well were inoculated with 50 μl of CCK-8 prepackaged solution. The plates were processed as described above, and the OD_{450} of the supernatant was measured.

Each experiment was performed in quadruplicate on at least two separate days, and data shown in the figures are means from one representative experiment. A statistical analysis was performed using StatView software (version 5.0.1; SAS Institute, Cary, N.C.).

Previous studies have not reported an actual relationship between *Candida* cell number and XTT formazan signal, nor have the XTT metabolic activities of different species and strains been compared. Differences in XTT signal have instead been assumed to be due to changes in cell number. To address this paucity of information, as well as to determine whether the XTT methodology could be applied more appropriately to future efforts at biofilm quantitation, we studied the relationship between inoculum size and XTT signal among different *C. albicans* and *C. parapsilosis* strains.

As shown in Fig. 1A, while there was a linear dose-response relationship between cell number and signal when the 1× concentration of XTT was used, *C. albicans* exhibited a steeper response curve than did *C. parapsilosis*. This is not a result of the XTT technique alone, since similar behavior was seen when WST-8 was used (Fig. 2). Such differences in the ability to metabolize tetrazolium salts persisted in the face of experimental variations of the concentrations of tetrazolium used (see below) and the incubation times and testing of additional species such as *Candida glabrata* (data not shown). Thus, the relationship between cell number and XTT signal cannot be assumed to be constant across *Candida* species.

Varying the XTT concentration (e.g., in an attempt to increase test sensitivity for species such as *C. parapsilosis*) may produce unexpected results. As shown in Fig. 1B, while *C. albicans* (strains M61 and GDH) showed predictable increases in OD, the two *C. parapsilosis* strains exhibited very different responses, with strain P/A71 showing a profound increase in signal at an inoculum of 10^8 blastoconidia. Thus, the yeast response to different tetrazolium concentrations is also strain dependent. Expected (proportional) differences in signal due to increasing XTT concentrations may in fact occur only at high cell concentrations.

Despite the widely published assumption that the XTT formazan product is water soluble and thus readily diffuses out of cells, we noticed during earlier work that there was a slight residual orange discoloration of *Candida* biofilms after washing, although the amount of retained dye was insignificant when assayed after acidified ethanol or DMSO extraction ($P > 0.05$) (data not shown). As shown in Fig. 3, this amount may be more significant when planktonic cells are assayed. With

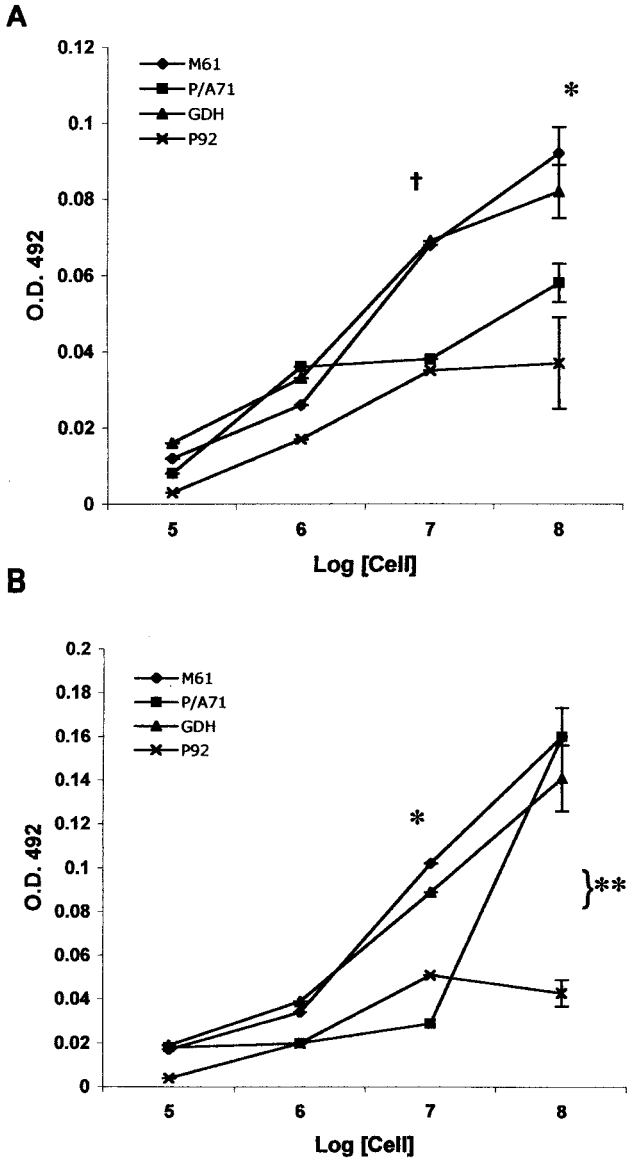

FIG. 1. (A) XTT formazan signal (measured at 492 nm) produced by *C. albicans* (strains M61 and GDH) and *C. parapsilosis* (strains P/A71 and P92) at concentrations ranging from 10^5 to 10^8 cells/ml, using 1-mg/ml (A) and 5-mg/ml (B) XTT stock solutions. The cross indicates a P value of <0.05 for results obtained with M61 and GDH compared with those obtained with P/A71 and P92; a single asterisk indicates a P value of <0.05 for all comparisons except M61 versus GDH; and a double asterisk indicates a P value of <0.05 for results for all strains versus that for P92. Error bars indicate ±1 standard deviation (SD); for clarity, only bars for 10^8 cells/ml are shown.

DMSO extraction of planktonic cell pellets, the signal intensity for both *C. albicans* and *C. parapsilosis* doubled at higher blastoconidia concentrations (10^8 cells/ml). Similar results were seen when the 5× concentration of XTT was used (data not shown).

The XTT assay and other colorimetric methods remain valuable tools for examining the behavior of yeast, whether in planktonic (10, 11) or biofilm form (2, 4, 5, 8, 9). Tetrazolium assays are increasingly being used to make direct comparisons between *Candida* isolates, sometimes in the absence of other

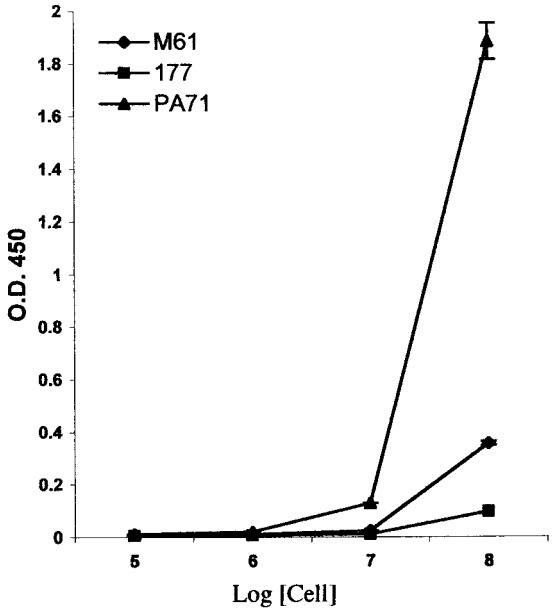

FIG. 2. WST-8 signal (measured at 450 nm) produced by *C. albicans* (strain M61) and *C. parapsilosis* (strains P/A71 and P177). Error bars represent ±1 SD; for clarity, only bars for 10^8 cells/ml are shown.

numerical methods (4, 6). Our group's earlier work suggested that this may not always be appropriate (8, 9). Here, we attempted to ascertain the uses and limits of the XTT assay in the study of different *Candida* isolates. Several conclusions can be drawn from our results. First, while tetrazolium assays are valuable for quantitation within a yeast strain, it cannot be assumed that there is necessarily a linear relationship between organism number and colorimetric signal. Second, one cannot make interstrain comparisons in the absence of detailed standardization, since different strains metabolize substrate with different capabilities. Third, the relationship between the XTT concentration used and the resultant colorimetric signal is not necessarily proportional; valid quantitation can only be performed after the creation of appropriate standard curves for each amount of tetrazolium used. Fourth, while the XTT formazan product readily appears in solution, there can be in some strains a significant amount of retained intracellular product, which only becomes soluble after cell treatment with DMSO. The amount of retained product may vary between different cellular states, e.g., planktonic and biofilm. In summary, studies attempting to make quantitative comparisons of the behavior of different strains of *Candida* need to assess variations in XTT metabolism. Solubilization of the XTT formazan product is likely to be an important step for planktonic cell assays.

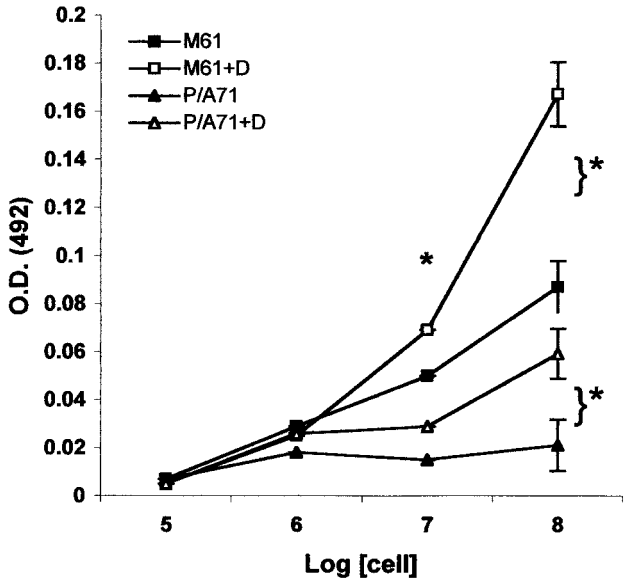

FIG. 3. XTT formazan signal (measured at 492 nm) produced by *C. albicans* (strain M61) and *C. parapsilosis* (strain P92) at concentrations ranging from 10^5 to 10^8 cells/ml, using a 1-mg/ml XTT stock solution. Values are one-half of standard due to 1:2 dilution with either DMSO (D) or PBS. A single asterisk indicates a P value of <0.05 for comparison of results for untreated and DMSO-treated cells. Error bars represent ±1 SD; for clarity, only bars for 10^8 cells/ml are shown.

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